Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice

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The establishment of clonal infection of hepatitis C virus (HCV) in a small-animal model is important for the analysis of HCV virology. A previous study developed models of molecularly cloned genotype 1a and 2a HCV infection using human hepatocyte-transplanted chimeric mice. This study developed a new model of molecularly cloned genotype 1b HCV infection. A full-length genotype 1b HCV genome, HCV-KT9, was cloned from a serum sample from a patient with severe acute hepatitis. The chimeric mice were inoculated intrahepatically with in vitro-transcribed HCV-KT9 RNA. Inoculated mice developed viraemia at 2 weeks post-infection, and this persisted for more than 6 weeks. Passage experiments indicated that the sera of these mice contained infectious HCV. Interestingly, a similar clone, HCV-KT1, in which the poly(U/UC) tract was 29 nt shorter than in HCV-KT9, showed poorer in vivo infectivity and replication ability. An in vitro study showed that no virus was produced in the culture medium from HCV-KT9-transfected cells. In conclusion, this study developed a genetically engineered genotype 1b HCV-infected mouse. This mouse model will be useful for the study of HCV virology, particularly the mechanism underlying the variable resistance of HCV genotypes to interferon therapy.

INTRODUCTION

Hepatitis C virus (HCV), a positive-sense, single-stranded RNA virus, infects and replicates efficiently only in the hepatocytes of humans and chimpanzees. There are many genotypes of HCV distributed worldwide (Simmonds et al., 1993); among them genotype 1b is the major genotype in Asia, including Japan, and is known to be one of the most resistant genotypes to interferon (IFN) therapy (Fried et al., 2002). Until recently, studies of HCV replication have long been hampered by the lack of a virus culture system. The development of HCV replicon systems has allowed the
study of the mechanisms of replication of HCV (Lohmann et al., 1999). However, these replicons lack structural proteins, do not replicate efficiently without adaptive mutations and do not produce infectious virions. Recently, it was reported that the genotype 2a full-length JFH-1 genome replicated efficiently in Huh7 cells without adaptive mutations and produced virions that were infectious for both naïve cells and chimpanzees, as well as for a human hepatocyte-transplanted chimeric mouse (Wakita et al., 2005; Zhong et al., 2005; Lindenbach et al., 2006). To date, five full-length genotype 1b clones, HCV-N (Beard et al., 1999), Con-1 (Bukh et al., 2002), HCV-J4 (Okamoto et al., 1992), HCV-CG1B (Thomson et al., 2001) and HCV-BK (Takamizawa et al., 1991), have been demonstrated to be infectious by intrahepatic inoculation of transcribed HCV RNA into the liver of chimpanzees. Among these, only the HCV-CG1B genome is reported to produce HCV particles when transfected into Huh7 cells (Heller et al., 2005).

Although the chimpanzee is a useful animal model for the study of HCV infection, there are ethical restrictions on the use of this animal. Instead, Mercer et al. (2001) developed a useful small-animal model for the study of HCV infection using chimeric urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) mice (which are immunodeficient and undergo liver failure) with engrafted human hepatocytes. This HCV-infected mouse model is reported to be useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease (Kneteman et al., 2006). We have previously described methods to improve the replacement levels of human hepatocytes in this mouse model (Tateno et al., 2004) and we have developed a reverse genetics system for hepatitis B virus (Tsuge et al., 2005) and HCV (Hiraga et al., 2007). In the present study, we report the establishment of an infectious genotype 1b HCV clone that infects and replicates efficiently in human hepatocyte chimeric mice.

**METHODS**

**Cloning of infectious genotype 1b HCV isolate.** Serum samples were obtained from a 43-year-old physician who developed severe acute hepatitis after needle stick exposure from a patient with chronic hepatitis C. On admission, the serum total bilirubin concentration was 10.0 mg dl⁻¹ and the prothrombin time was 40%. The patient tested positive for HCV antibodies by a third-generation radioimmunoassay (Ortho-Clinical Diagnostics) and for HCV RNA by RT-PCR. Serum HCV RNA was quantified using an Amplicor Monitor HCV test (Ortho-Clinical Diagnostics). The HCV RNA titre was 2.5 x 10⁶ copies ml⁻¹ on admission and then decreased gradually. Fig. 1 shows the serial changes in alanine aminotransferase (ALT) as a measure of liver function and HCV RNA levels in this patient. Serum samples obtained in the early phase of infection were used for cloning the full-length genome.

**RNA extraction, cDNA synthesis, plasmid construction and RNA transcription.** Total RNA was extracted from 100 μl serum samples using SepaGene RV-R (Sanko Jyunyaku) and reverse transcribed with random hexamers and ReverTra Ace reverse transcriptase (Toyobo) according to the manufacturer’s instructions. PCR primers were designed based on the sequence of HCV-Con1 (GenBank accession no. AI238799; Bukh et al., 2002). Five overlapping cDNA segments (nt 1–2292, 2269–6715, 6696–9094, 7564–9404 and 9361–9605; nucleotide numbers are those of HCV-Con1) were amplified by PCR with TaKaRa LA Taq polymerase (Takara Biochemicals) using the above cDNA. Amplified products were separated by agarose gel electrophoresis. Nucleotide sequences were determined using a Big Dye Terminator Mix Cycle Sequencing kit (Applied Biosystems Japan) with an automated DNA sequencer (model 310; PE Biosystems). We corrected the nucleotide sequences of the obtained clones by site-directed mutagenesis and made them identical to the nucleotide sequences obtained by direct sequencing. Naturally occurring restriction enzyme cutting sites were utilized to clone each segment. We utilized the vector pBR322 and created a multiple-cloning site under the control of the T7 promoter by ligating a linker at restriction enzyme cutting sites as they appeared in the HCV sequences (Fig. 2a). Each segment of HCV was cloned into this vector to generate the full-length clones. The HCV-KT9 clone was established using the 3'-terminal fragment with the longest poly(U/UC) tract length (115 nt), which should have a high replication ability (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). A clone with a shorter poly(U/UC) tract length (86 nt), HCV-KT1, was also generated. A polymerase-deficient mutant with an amino acid substitution in the GDD motif (GDD→I) was generated using a Quick Change Site-Directed Mutagenesis kit (Strategene). After digesting the plasmid with XbaI (New England BioLabs) at the 3' end of the HCV cDNA, HCV RNA was transcribed using T7 RNA polymerase (MEGAAscript; Ambion) at 37 °C for 3 h in a 100 μl reaction mixture, according to the manufacturer’s instructions. The RNA was analysed using denaturing agarose gel electrophoresis and kept at −80 °C until use.

**Construction of a phylogenetic tree.** A phylogenetic tree was constructed based on the entire nucleotide sequences of 26 full-length genotype 1b clones plus HCV-KT9. The total number of synonymous and non-synonymous substitutions among the nucleotide sequences was estimated using the method of Gojobori et al. (1982) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987).

![Graph showing clinical course of patient with severe acute hepatitis C. Alanine aminotransferase (ALT) and prothrombin time (PT) are shown from the day of admission (day 1). The patient was treated daily with 10⁶ U IFN-α intravenously for 5 days, followed by 10⁶ U IFN-α intramuscularly three times a week for 6 months. HCV RNA was measured on days 1, 7, 13 and 17 (arrowheads). A serum sample was taken on day 1 (arrow) and used to clone the full-length HCV genome.](http://vir.sgmjournals.org)
Intrahepatic injection experiments in human hepatocyte chimeric mice. We used methods described previously (Tateno et al., 2004) to generate uPA+/+/SCID−/+ mice and transplant human hepatocytes. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index and were measured as described previously (Tateno et al., 2004). Intrahepatic injection of RNA, extraction of serum samples and euthanasia were performed under ether anaesthesia. Briefly, 500 μl RNA solution containing 30 μg transcribed HCV RNA was injected into the liver of anaesthetized chimeric mice through a small abdominal incision. RNA extraction from mouse serum samples, quantification of HCV RNA and nested PCR were performed as described previously (Hiraga et al., 2007). All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments and under the approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Cell culture, RNA transfection and measurement of HCV core antigen. The human hepatoma cell line Huh7 was maintained in Dulbecco’s modified Eagle’s medium (Sigma) containing 10 % fetal calf serum. RNA transfection and measurement of HCV core antigen in the culture medium were performed as described previously (Wakita et al., 2005).

Statistical analysis. The infectious ratio of chimeric mice was compared and the differences assessed using a χ² test. Differences in HCV RNA replication ability in vitro were analysed statistically by one-way analysis of variance followed by Scheffe’s test. A P value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of genotype 1b clones HCV-KT9 and HCV-KT1

The entire genome of HCV cDNA was assembled from five DNA fragments (Fig. 2a). We obtained 24 3′-extremity clones with different poly(U/UC) tract lengths. We selected the clone with the longest (U/UC) tract because a previous study indicated that the length of poly(U/UC) tract correlates with HCV replication in an HCV replicon system (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). The length of the poly(U/UC) tract in the longest 3′ clone was 115 nt. The entire genome length of the HCV-KT9 clone using this longest 3′ clone was 9621 nt. We also generated the clone HCV-KT1 with a shorter (86 nt) poly (U/UC) tract to compare the replication abilities of these clones. The lengths of the poly(U/UC) tracts of 22 clones deposited in GenBank are shown in Fig. 2(b). All infectious clones had a poly(U/UC) tract longer than 80 nt. Fig. 2(c) shows a phylogenetic tree constructed using the nucleotide sequences of the 26 full-length genotype 1b clones published to date. Interestingly, the sequence of HCV-KT9 was closest to that of HCV-CG1b (GenBank accession no. AF333324), which has been reported to be infectious, and formed a cluster with two other infectious clones, HCV-N (Beard et al., 1999) and HCV-BK (Takamizawa et al., 1991). We compared the amino acid...
sequences of HCV-KT9 with an alignment of the sequences of the 26 other genotype 1b strains. All HCV full-length clones reported from Japan were included in these 26 strains. Based on these comparisons, we identified 25 aa unique to HCV-KT9 (Fig. 2a). We found that the amino acid sequence of the IFN sensitivity-determining region in the NS5A region, which has been suggested to mediate IFN resistance via interaction with the cellular protein kinase R (Enomoto et al., 1996; Gale et al., 1997), was that of the wild-type.

Intrahepatic injection of HCV-KT1 and HCV-KT9 RNAs into human hepatocyte chimeric mice

In the next experiments, 30 µg in vitro-transcribed RNA of HCV-KT1, HCV-KT9 or HCV-KT9-GND was injected into the livers of chimeric mice. Eight of 10 (80%) HCV-KT9-injected mice developed measurable viraemia at 2 weeks post-inoculation (Table 1 and Fig. 3), with the HCV RNA titre reaching 1.1 × 10^6 to 8.8 × 10^6 copies ml^-1 at 6 weeks post-inoculation (Fig. 3). To check for the presence of infectious HCV in the serum of HCV-KT9-infected mice, each of five naïve mice was injected with 10 µl serum sample (containing 3.5 × 10^5 copies of HCV) obtained from an HCV-KT9-infected mouse 6 weeks after inoculation. All five naïve mice became positive for HCV RNA, as confirmed by nested PCR, at 2 weeks post-infection and two mice developed persistent viraemia (Fig. 4). These results indicated that HCV-KT9 replicates efficiently in mice livers and produces infectious virus continuously. On the other hand, only one out of seven HCV-KT1-injected mice (14%) developed measurable viraemia (Table 1 and Fig. 3). The level of viraemia was low in this HCV-KT1-injected mouse, HCV RNA was negative by nested PCR at 2 weeks after inoculation and the titre was only 2.2 × 10^4 copies ml^-1 at 4 weeks post-inoculation (Fig. 3). These results confirmed the importance of the poly(U/UC) tract length in experimentally induced viraemia.

The nucleotide and amino acid sequences of the viral genome isolated from an HCV-KT9-injected mouse (Fig. 3)

Table 1. Correlation between length of the poly(U/UC) tract and HCV infection

<table>
<thead>
<tr>
<th>Clone</th>
<th>Length of poly(U/UC) tract</th>
<th>Number of mice</th>
<th>Infection ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-KT1</td>
<td>86</td>
<td>1/6/7</td>
<td>14%</td>
</tr>
<tr>
<td>HCV-KT9</td>
<td>115</td>
<td>8/2/10</td>
<td>80%*</td>
</tr>
<tr>
<td>HCV-KT9-GND</td>
<td>115</td>
<td>0/3/3</td>
<td>0%</td>
</tr>
</tbody>
</table>

*P=0.015, compared with HCV-KT1.

at 6 weeks after RNA injection were identical to the injected HCV-KT9 (data not shown). We tried to reclone the poly(U/UC) tract in the HCV-KT1-infected mouse, but it was impossible to reamplify the HCV cDNA using the remaining small amount of serum.

Analysis of virus production from HCV-KT9-transfected cells

Next, we evaluated the ability of the HCV-KT9 clone to replicate in transfected HuH7 cells. In these experiments, we used JFH-1 RNA, which is known to replicate efficiently in cell cultures, as control (Wakita et al., 2005). Core protein was secreted efficiently from JFH-1 RNA-transfected HuH7 cells. In contrast, we did not observe any measurable levels of core protein in the supernatant of HCV-KT9-transfected cells (Fig. 5), suggesting a minimal replication ability of HCV-KT9 to produce and release virus into the supernatant.

DISCUSSION

In this study, we described the establishment of a genotype 1b clone, HCV-KT9, that replicated efficiently following injection of the transcribed RNA into chimeric mouse liver.
The key factor that determines the infectivity of HCV clones has not yet been established. We previously established a clone from HCV that replicated in a chimeric mouse after injection of serum from a chronically HCV-infected patient. However, we did not observe viraemia after intrahepatic injection of the transcribed RNA from this clone (unpublished results). In contrast, injection of HCV-KT9 RNA in the present study resulted in viraemia in eight out of ten mice (80%). The fact that the nucleotide and amino acid sequences of the virus recovered from the infected mice were identical to those of the HCV-KT9 clone indicated that no adaptive mutation was necessary for this clone to replicate in the chimeric mouse.

Interestingly, the clone was obtained from a patient with severe acute hepatitis. This is similar to JFH-1, an HCV clone with a strong replication ability in cultured cell lines, chimpanzees and chimeric mice, which was cloned from serum samples of a patient who developed acute fulminant hepatitis with a high virus titre (Wakita et al., 2005). A virus that replicates in the early stage of infection may have strong replication ability, which may be lost in the chronic phase of infection.

A key amino acid substitution may be present in one (or some) of the amino acids unique to this clone (Fig. 2a). We also showed that clone HCV-KT1, which differs from HCV-KT9 only in the length of the poly(U/UC) tract, had a poorer replication ability in mice (Table 1 and Fig. 3). However, there is a possibility that a shorter poly(U/UC) tract only slows down the rate of infection, as the HCV RNA titre in the HCV-KT1-infected mouse at 6 weeks after inoculation was similar to that in HCV-KT9-infected mice (Fig. 3). It has been reported that the length and composition of the poly(U/UC) tract is important for the replication of HCV replicons (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). However, no replication advantage of a poly(U/UC) tract longer than 86 bp was revealed in this study. This may be due to differences in vitro and in vivo, where the innate immune response against the virus may be more robust than in cell culture.

As shown in the present study, reverse genetics of HCV has become available for studies of HCV replication. The important factors for virus replication suggested above can be analysed further using this system.

We also examined the response of HCV-KT9-infected mice to IFN treatment. Three HCV-KT9-infected mice were treated with daily intramuscular injections of 1000 IU IFN-α (g body weight)^−1 for 2 weeks. This regimen resulted in a reduction in HCV RNA levels of only 1.0 log copies ml^−1 (data not shown). These results are consistent with our previous study, which showed a similar low-level reduction in HCV RNA in mice infected with a genotype 1a clone, and differ from our previous results in mice infected with HCV genotype 2a, which became negative for HCV RNA following daily treatment with 1000 IU IFN-α (g body weight)^−1 for 2 weeks (Hiraga et al., 2007). These results are in agreement with our clinical experience that genotype 1 is more resistant to IFN therapy than genotype 2. As shown in the present study and previously (Hiraga et al., 2007), reverse genetics of HCV with three genotypes, 1a, 1b and 2a, is now available. By recombination of these clones or the establishment of mutants with nucleotide and amino acid sequences similar to each other, it may be possible to clarify the mechanism underlying the variability in susceptibility of HCV genotypes to IFN.

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**Fig. 4.** Passage experiments of HCV in naïve chimeric mice. Five naïve chimeric mice were inoculated intravenously with 10 µl serum sample (containing 3.5×10^6 copies HCV) obtained from an HCV-KT9-infected mouse at week 6 post-inoculation. Serum samples were obtained at the indicated time intervals for the measurement of HCV RNA levels and HSA concentrations. Data represent the changes in five individual mice.

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**Fig. 5.** Time-course studies of HCV core protein secretion into the culture medium of HCV RNA-transfected cells. Huh7 cells were transfected with 10 µg HCV-KT9, HCV-KT9-GND or JFH-1 RNA. HCV core antigen in the culture medium was measured at 24, 48 and 72 h after transfection. Data are shown as mean ± SD of HCV core protein levels obtained from three independent transfection experiments.

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In this study, HCV-KT9 showed no virus production ability in vitro. Recently, Kato et al. (2007) reported that the genotype 1b HCV clone CG1b replicated in Huh7.5.1 cells and produced infectious HCV. It will be of interest to the genotype 1b HCV clone CG1b replicated in Huh7.5.1 mice. This model will be useful for studies of HCV replication, particularly the mechanism underlying the variable resistance of HCV genotypes to IFN therapy.

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